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C-type lectin receptor expression on human basophils and effects of allergen-specific immunotherapy

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Short title: Immunotherapy alters CLR expression on basophils.

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Abbreviations

AIT – Allergen-specific immunotherapy

CLEC - C-type lectin domain family

CLECSF - C-type lectin superfamily member

CLR - C-type lectin receptor

DCIR - Dendritic cell immunoreceptor

DC-SIGN - Dendritic cell-specific ICAM-3-grabbing non-integrin 1

Dectin - Dendritic cell-associated C-type lectin

Der f - *Dermatophagoides farinae*

Der p - *Dermatophagoides pteronyssinus*

FcR – Fc receptor

FMO – Fluorescence minus one

HDM – House dust mite

ITAMs - Immunoreceptor tyrosine-based activation motifs

MR - Macrophage mannose receptor, C type 1

MRC2 - Mannose receptor, C type 2

PAMP - Pathogen-associated molecular patterns

PBMC - Peripheral blood mononuclear cells

PRR – Pattern-recognition receptor

TLR - Toll-like receptor

Abstract

Basophils are emerging as immunoregulatory cells capable of interacting with their environment not only via their characteristic IgE-mediated activation, but also in an IgE-independent manner. Basophils are known to express and respond to stimulation via TLR2, TLR4, DC-SIGN and DCIR, but whether basophils also express other C-type lectin receptors (CLRs) is largely unknown. In this study, we investigate the CLR expression profile of human basophils using multi-color flow cytometry. Since FcRs as well as some CLRs are associated with allergen recognition and shown to be involved in subsequent immune responses, the expression of CLRs and FcRs on peripheral blood basophils, as well as their frequency, was monitored for one year in subjects undergoing subcutaneous allergen-specific immunotherapy (AIT). Here, we show that human basophils express CLECSF14, DEC205, Dectin-1, Dectin-2 and MRC2. Furthermore, we demonstrate that the frequencies of basophils expressing the allergy-associated CLRs Dectin-1 and Dectin-2 were significantly reduced after one year and eight weeks of AIT, respectively. In contrast, the frequency of basophils positive for Fc γ R2, as well as the fraction of total basophils, significantly increased after one year of AIT. The herein demonstrated expression of various CLRs on basophils, and their altered CLR and FcR expression profile upon AIT, suggest yet unexplored ways by which basophils can interact with antigens and may point to novel immunoregulatory functions targeted through AIT.

Keywords

allergen-specific immunotherapy (AIT), allergy, basophils, C-type lectin receptors (CLR), Fc receptors (FcR), pattern recognition receptors (PRR), Toll-like receptors (TLR)

Introduction

Basophils are well known for their role as IgE-mediated effector cells in allergic reactions. In addition, recent studies have shown that human basophils have immunomodulatory roles and can respond to antigens by IgE-independent mechanisms, e.g. via Toll-like receptors (TLRs) [1,2]. TLRs are important pattern-recognition receptors (PRRs) that recognize pathogen-associated molecular patterns (PAMPs) on the surface of various microbes [3]. Upon stimulation of TLR1 to TLR9, human basophils release cytokines, e.g. IL-4, IL-8, IL-13, RANTES and BAFF, without inducing degranulation [2,4,5], and the nature of these cytokines depends on the TLR triggered [2]. For instance, stimulation of TLR2 with heat-killed *Listeria monocytogenes* leads to the production of IL-8 by basophils, while LPS-stimulation via TLR4 generates IL-8 and IL-13 [2]. Thus, basophils selectively regulate the immune system in response to different microbes.

In addition to TLRs, C-type lectin receptors (CLRs) are essential PRRs that are expressed by several immunologically relevant cell types, such as dendritic cells and macrophages [6–12]. Depending on the cell type, receptor and ligand, the CLRs exert distinct functions. For example, DEC205/CD205, the Macrophage mannose receptor C type 1 (MR)/CD206 and Mannose receptor C type 2 (MRC2)/CD280 can mediate antigen uptake [8,9,12], whereas CLRs such as the C-type lectin superfamily member 14 (CLECSF14)/CD301, Dendritic cell-associated C-type lectin 1 (Dectin-1) and Dectin-2, directly trigger intracellular signaling pathways that subsequently affect the immune response [6,7,11]. However, current knowledge on the involvement of various CLRs on human basophils in the recognition of PAMPs is very limited, and there is to our knowledge only one study, which was recently published, that shows the expression and function of CLRs on basophils [13]. Jiang *et al.* showed that human basophils express the CLRs Dendritic cell-specific ICAM-3-grabbing non-integrin 1 (DC-SIGN) and Dendritic cell immunoreceptor (DCIR) and that they via these

receptors aid in the spreading of HIV-1 particles to CD4⁺ T cells [13]. These results support that CLRs on basophils play an important role for pathogen-selective responses.

Several PRRs are also shown to bind to allergens and trigger allergic immune responses [14–16]. MR, DC-SIGN, TLR4 and TLR5 as well as Dectin-1 and Dectin-2 are all receptors that have been associated with allergens and are suggested to transmit signals for generation of allergic responses, as reviewed by Salazar *et al.* [17,18]. Interestingly, human basophils are known to express TLR2, TLR4 and DC-SIGN on their surface [4,13,19], and the human basophilic cell line KU812 was recently shown to express TLR5 [20]. Additionally, KU812 respond to the house dust mite (HDM) allergen *Dermatophagoides pteronyssinus* (*Der p*) 1 by producing IL-4, IL-5 and IL-13 in a proteolytic manner involving undefined IgE-independent mechanisms [21]. Furthermore, a synergistic effect on cytokine production was seen when IgE was cross-linked in parallel with stimulation of either TLR4 or TLR9 on basophils [2]. Basophils are hence involved in IgE-independent responses towards allergens that could play a part during both the allergen sensitization and in the effector phase. These results suggest that insights into basophil expression of allergy-associated PRRs can increase our understanding of allergic immune responses.

Currently, the only curative treatment that can induce long-term clinical improvement against allergic diseases is allergen-specific immunotherapy (AIT). However, the underlying mechanisms are not completely understood. Immunological changes known to be associated with the beneficial effects of AIT comprise a switch from Th2 to Th1 cytokines and an increase in functional regulatory T cells, as recently reviewed [22]. In addition, basophils decrease their ability to degranulate and to cause systemic anaphylaxis already during the first day of treatment [23]. Furthermore, AIT leads to immunoglobulin class switching from IgE to IgG1 and IgG4 [24], which subsequently can compete with IgE for allergen binding [25], and thereby prevent FcεRI-mediated activation of basophils and mast cells. Basophils

are hence affected by the availability of IgG4 and IgE but it is currently unclear whether basophils alter their expression of PRRs and Fcγ receptors (FcγRs) during AIT. Such changes could give further insights into the mechanisms of AIT.

In this study, we outline the PRR and Fc receptor (FcR) expression profile of human basophils, as well as investigate AIT-induced alterations of the basophil phenotype and frequency. In this context, the first year of AIT for allergic rhinitis was used as an experimental model with the aim of identifying the expression of twelve PRRs, as well as two FcRs, i.e. FcγRI (CD64) and FcγRII (CD32) on human basophils. We demonstrate that human basophils express the CLRs CLECSF14, DEC205, Dectin-1, Dectin-2 and MRC2 on their surface. Additionally, the data presents that the fraction of basophils expressing Dectin-2 significantly decreases after eight weeks of AIT. Similarly, a statistically significant decrease in the fraction of basophils expressing Dectin-1 is seen after one year of AIT, while one year of AIT significantly increases the percentage of FcγRII expressing basophils. By assessing the PRR and FcR profile of human basophils we expand our knowledge about basic immunological mechanisms involved in allergic responses, which could lead to improved tools for predicting the outcome of AIT as well as the development of improved and safer therapeutics.

Materials and Methods

Donors and blood sampling procedure

13 allergic subjects and 6 non-allergic subjects were included in the study. Of the allergic subjects, 7 were followed during AIT while 6 were not qualified for AIT. Sensitizations were determined by skin prick test and are, together with the allergen mixtures used for AIT, summarized in Table 1. A standard clinical protocol, where increasing doses of allergens

were injected subcutaneously, was used for AIT. Blood samples from allergic subjects undergoing AIT were donated at three different time points; before AIT started (t0), after approximately 8 weeks of AIT (t8w) when the subjects had reached a dose of 10.000 standardized quality (SQ) units (ALK-Abelló A/S, Hørsholm, Denmark) and finally after one year of AIT (t1y) when the subjects had received the maximum dose (100.000 SQ units) for about six months. Blood was donated 7-10 days after the last allergen administration. The immunotherapy study was initiated in the autumn; consequently, all three time points occurred outside of the pollen season, when the donors were not exposed to environmental pollen allergens and they did not medicate. Serum samples were collected and examined for their allergen-specific IgE and IgG4 levels with Immucap (Thermo Scientific, Allerod, Denmark) at the three time points. The allergic subjects not undergoing AIT and the non-allergic donors were sampled around t0 and t8w, respectively. Except for their allergy, all subjects were considered healthy. The regional Ethics Committee approved the study (Dnr 2010/321) and written informed consent was obtained from all donors.

Cell preparations and antibody staining

Peripheral blood mononuclear cells (PBMC) were prepared from heparinized blood, immediately after sampling, by density gradient centrifugation using Lymphoprep (Medinor, Lidingö, Sweden). The PBMCs were centrifuged and washed twice. Basophils were identified as lineage (CD3, CD14 and CD19) and HLA-DR negative cells, positive for CD123 (Figure 1a). A single PRR or FcR was measured per sample and, as recommended for accurate flow cytometry data interpretation [26], an identical fluorescence minus one (FMO) control was included for each receptor. Antibodies used include: CD14-PE, CD19-PE, CD19-FITC (Dako Cytomation, Glostrup, Denmark), CD123-biotin, CD123-PE, CD3-PE, CD3-FITC, FcγRII/CD32-FITC, SA-APCCy7 (BD Biosciences, San Jose, CA), HLA-DR-PerCPCy5.5, TLR2-PE, TLR4-PE (Biolegend, San Diego, CA), CD14-FITC (Invitrogen,

Carlsbad, CA), CLECSF14/CD301-Alexa fluor 488 (Dendritics, Lyon, France), MR/CD206-PE, Langerin/CD207-PE (Beckman Coulter, Brea, CA), DEC205/CD205-FITC (eBioscience, San Diego, CA), Dectin-1-FITC (AbD Serotec, Kidlington, UK), FcγRI/CD64-PE, Dectin-2, DCIR, C-type lectin domain family 9a (CLEC9a) (R&D Systems, Minneapolis, MN), MRC2/CD280 and DC-SIGN/CD209 (Abcam, Cambridge, UK). Unconjugated CLR-antibodies were detected using either rabbit anti-mouse-PE (Dako Cytomation) or goat anti-rabbit-FITC (BD Biosciences). A strict staining procedure was followed with extra care taken to ensure exact antibody concentrations for each time point. Mouse IgG blocking antibodies were included to prevent unspecific staining, either before any antibodies were added or, if indirect stainings were performed, after the secondary staining. The same antibodies were used for all three time points except for the antibodies that expired prior to the final analysis. In these cases the new lot was compared to the old one to ensure equal staining. Unfortunately, the antibodies used to analyse TLR2 and TLR4 displayed reduced binding capacity to their target receptor at the t1y time point, and these markers were therefore excluded from AIT analysis over time. BD FACS Canto II (BD Biosciences) was used for flow cytometer analysis and according to instrument guidelines the BD Cytometer Setup & Tracking beads (BD Biosciences) were used daily. Additionally, application settings were applied to ensure equal performance of the instrument at every analysis.

Analysis

FCS Express version 4 (De Novo Software, Los Angeles, CA) was used for the expression analysis. Basophils were gated as described (Figure 1a) and the threshold for each PRR and FcR was set based on the corresponding FMO control sample (Figure 1b), which contained all antibodies except the one for which the threshold should be determined. FMO controls are

considered better controls than isotype controls in multicolor experiments as the background due to spillover from other fluorescence conjugates generally is more considerable than unspecific binding [27]. Results are displayed as net values for the stained samples minus the FMO controls (i.e. % positive cells of stained sample minus % positive cells in FMO sample), and negative values were set to zero. The number of donors included in each PRR and FcR analysis can be seen in Supplementary Table S1. Generally, 10-13 allergic and 5-6 non-allergic subjects were analysed at t0. For the majority of assessments during AIT 6-7 donors were analysed. In the analysis of basophil frequency 7 donors were analysed during AIT. The reason for not showing results from all donors for all receptors is that the statistical test required data from all three time points. This was not possible in all cases due to; i) the receptor-specific antibodies were not available at all time points and could not be replaced due to lot variations, and ii) blood was not received from one donor at one time point. GraphPad Prism version 6 (GraphPad Software, San Diego, California, USA) was used for the statistical analyses. Comparisons over time in the AIT study were calculated using Friedman test followed by Dunn's multiple comparison test, while comparisons between healthy and allergic subjects were performed using Mann-Whitney test. P values of less than 0.05 were considered statistically significant.

Results

Human peripheral blood was obtained from 6 non-allergic subjects and 13 allergic subjects, of whom 7 were included in the AIT study and 6 were non-AIT subjects. PBMC were prepared immediately after collection, whereupon basophils were analysed with flow cytometry and gated as indicated in Figure 1a. The AIT subjects were immunized with personalized mixtures of allergen extracts (Table 1). Peripheral blood from the AIT subjects

was collected, in addition to before the AIT study (t0), also at two more time points during the first year of AIT; after eight weeks (t8w) and one year (t1y). Serum was obtained from the AIT subjects and consistent with literature [24,28], the concentrations of allergen-specific IgG4 increased in serum already at t8w of AIT for all donors except one and increased even further in measurements at t1y of therapy (Supplementary Figure S1). Additionally, the allergen-specific IgE levels showed an initial increase at t8w, which subsequently decreased for the majority of donors at t1y of AIT (Supplementary Figure S1). A control group with subjects clinically qualified for AIT treatment receiving sham injections was not included due to ethical reasons.

Human basophils express CLECSF14, DEC205, Dectin-1, Dectin-2 and MRC2

The cells from allergic and non-allergic donors were analysed with multi-color flow cytometry, and FMO controls were used (Figure 1b) to carefully assess the surface expression of various PRRs and Fc-receptors on human basophils. No statistically significant difference in baseline surface expression was seen between the allergic and non-allergic subjects for any of the receptors; hence, the two groups were combined to summarize the basophil PRR and FcR expression profile (Figure 2a-c, Supplementary Table S1). The separate profiles for allergic and non-allergic subjects are presented in Supplementary Figure S2. Notably, the results show for the first time that basophils express multiple CLRs on their cell surface, i.e. CLECSF14, DEC205, Dectin-1, Dectin-2 and MRC2 (Figure 2a). In concordance with a previous study, we also demonstrate a high frequency of DC-SIGN and DCIR positive basophils [13]. In contrast, MR, Langerin/CD207 and CLEC9a were not expressed. Even though the frequency of basophils expressing TLR2 and TLR4 was very low (Figure 2b), a clear positivity was seen in several cases (Supplementary Figure S3), as previously demonstrated [4,19]. Additionally, high expression levels of Fc γ RII were detected on a large

proportion of basophils (Figure 2c). In contrast, only a small fraction of basophils expressed FcγRI.

AIT alters the CLR profile of human basophils

Human basophils were analysed at t0, t8w and t1y of AIT, to determine whether the PRR and FcR profiles were altered during AIT. Interestingly, statistically significant reductions were observed in the percentage of basophils expressing the allergy-associated CLRs Dectin-1 at t1y and Dectin-2 at t8w of AIT as compared to t0 (Figure 3a and 3b). Moreover, expression of Dectin-2 appeared to decrease on basophils from all subjects at t1y, although this decrease was not statistically significant, most likely due to the limited donor cohort. Furthermore, a statistically significant increase was seen for the frequency of basophils expressing FcγRII at t1y of AIT (Figure 3c). Expression of all CLRs and FcRs assessed during AIT is shown in Supplementary Figure S4, including those not showing significant alterations.

The frequency of basophils in peripheral blood increases after one year of AIT

The basophil frequency was determined by flow cytometry at t0, t8w and t1y, to assess whether the fraction of basophils in peripheral blood varied between non-allergic and allergic subjects, and whether it was altered during AIT. No statistically significant difference in the frequency of basophils in peripheral blood was seen when comparing the allergic to the non-allergic subjects at t0 (data not shown). In contrast, the results showed a significantly increased frequency of basophils in peripheral blood at t1y of AIT, and an increasing trend was seen already at t8w as compared to at t0 (Figure 4). These results indicate that AIT alters the relative amount of basophils in peripheral blood.

Discussion

This study, utilizing the first year of AIT for allergic rhinitis as an experimental model, expands the knowledge of the human basophil immunophenotype by outlining their expression of several PRRs and FcRs. We demonstrate for the first time surface expression of CLECSF14, DEC205, Dectin-1, Dectin-2 and MRC2 on basophils. Furthermore, the results show that the basophil frequency and CLR/FcR expression profile are affected during AIT.

Basophils are, as reviewed by Cromheecke *et al.*, able to respond to various IgE-dependent and IgE-independent ligands and participate in multiple immune responses by releasing cytokines and other mediators [29]. Thus, although human basophils do not appear to function as antigen-presenting cells in allergic reactions [30,31], it is clear that basophils have an immunomodulatory role. In addition to TLRs, CLRs are important PRRs influencing immune responses upon antigen recognition [32]. In this study, we demonstrate a high frequency of DC-SIGN and DCIR positive basophils, which is in concordance with a recently published article describing a functional role of these receptors in HIV-1 transfer to CD4⁺ T cells [13]. In addition, we show surface expression of Dectin-1, Dectin-2, DEC-205, CLECSF14, DC-SIGN and DCIR on basophils. The expression of various CLRs, presented in this study, suggests new and unexplored ways by which basophils can interact with antigens. Dectin-1 and Dectin-2, for example, are known to connect the innate and adaptive immunity by triggering Syk-pathways via immunoreceptor tyrosine-based activation motifs (ITAMs) [33]. Dectin-1 binds fungal β -glucans and can trigger Th1 and Th17 responses without pathogen internalization [7]. Thus, basophils may respond to Dectin-1 by intracellular signaling without internalization. Dectin-2 is mainly thought to recognize highly mannosylated structures expressed by fungi, and is critical for Th17 immunity activation in response to these pathogens [34]. Although CLECSF14 and Dectin-1 were expressed at low levels on basophils they might be able to respond to stimulation, as previously shown for

TLR2 and TLR4 that are known to induce responses despite low expression levels on basophils [2,4,5]. In summary, basophils are likely to respond to various pathogens based on the PRR profile outlined in this study.

The decrease in the expression of both Dectin-1 and Dectin-2 on basophils during AIT is interesting since these receptors, in addition to responding to fungal antigens as described previously, recently were suggested to interact with allergens [14,35–37]. For instance, β -glucans, which are present in HDM extract, were shown to induce CCL20 secretion by human airway epithelial cells in a TLR- and protease-independent, but Syk-dependent, manner, and Dectin-1 was therefore suggested to be involved in HDM allergen-recognition [37]. Additionally, Barrett *et al.* showed that activation of Dectin-2 on bone marrow-derived murine dendritic cells generate cysteinyl-leukotrienes in response to the HDMs *Dermatophagoides farinae* (*Der f*) and *Der p* as well as to the mold *Aspergillus fumigatus* [35]. Dectin-2 was also shown to be involved in the induction of Th2 immunity in response to *Der f*, and stimulation of Dectin-2 generated cytokines, including TNF, IL-6, IL-10 and IL-23 from bone marrow-derived murine dendritic cells [14]. Furthermore, murine alveolar macrophages can similarly sense HDM allergen via Dectin-2 and elicit production of cysteinyl-leukotrienes important for initiation of airway inflammation [36]. Notably, basophils are known to produce and release cysteinyl-leukotrienes when activated by IgE-mediated stimuli. Since Dectin-2 can trigger cysteinyl-leukotriene production in other cell types, it is possible that basophils are also capable of becoming activated and generate cysteinyl-leukotrienes and/or other mediators in response to Dectin-1 or Dectin-2 ligands. The reduced expression of Dectin-1 and Dectin-2 occurring during AIT suggests a reduced ability of basophils to respond to allergen stimulation via Dectin-1 and Dectin-2 upon AIT. The fact that differences were demonstrated despite the limited number of donors strengthens

that the phenotype of basophils is significantly altered by AIT, and opens up for a new field of investigations to understand the mechanisms behind AIT.

Noteworthy, the frequency of basophils expressing FcγRII was significantly upregulated during AIT in this study. The antibody used to determine FcγRII expression detected both FcγRIIA and FcγRIIB, and both of these are shown to be expressed by basophils [38]. The increased expression seen for FcγRII in this study is interesting as cross-linking of FcγRIIB with FcεRI can downregulate the stimulatory capacity of FcεRI, and FcγRIIA has furthermore been suggested to cooperate with FcγRIIB in the inhibition of basophil activation [38,39]. Shamji *et al.* showed that allergen-specific IgG4 not only inhibit basophil activation by competing with IgE for binding to allergens, but also by promoting FcγRII-mediated inhibition of FcεRI-triggered basophil activation [40]. Furthermore, Zidarn *et al.* recently demonstrated that although the allergen-specific IgG4 levels are reduced to pretreatment levels a couple of years after AIT, their inhibitory effect on basophil degranulation still remains [41], perhaps due to increased FcγRII expression. Taken together, it seems likely that an increased expression of FcγRII could be part of the mechanism behind the downregulated basophil activation occurring upon AIT.

In this study, a statistically significant increase in the peripheral blood basophil frequency was observed after one year of AIT. However, a lower relative amount of basophils in peripheral blood and tissue seem to correlate with health benefits [42–44]. For example, patients with allergic rhinitis get an increased frequency, and absolute cell count, of peripheral blood basophils during the entire pollen season, which correlate with increased seasonal symptom scores, while this is not observed for healthy subjects [42]. Additionally, allergic rhinitis patients experience an influx of basophils in the nose shortly after allergen challenge and a peak is reached after 6-24 hours, whereupon basophil numbers fall rapidly [43]. Also, a seasonal increase of basophils in nasal epithelium was shown to be partly

inhibited in grass pollen allergic patients after two years of successful AIT [44]. A possible explanation for the increased frequency of basophils in peripheral blood upon one year of AIT seen in this study is the fact that the AIT subjects have been exposed to a continuous high dose of allergen. The long term goal of AIT is to induce tolerance towards the allergen(s) used; however, previous studies have shown that more than two years of treatment generate better health improvements [45,46]. It might, hence, take more than one year for a new, stable basophil equilibrium to be reached.

In summary, we demonstrate for the first time that human peripheral blood basophils express multiple CLRs on their cell surface, and these results suggest many new ways in which basophils can interact with their environment. Additionally, the altered surface expression of Dectin-1, Dectin-2 and FcγRII during AIT, could make these receptors into useful biomarkers worth monitoring to follow the clinical response to AIT. This remains, however, to be verified in a larger, randomized and controlled trial setting.

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Authors' contributions

KL, LG and ML designed the study strategy. LG and ML wrote the ethical application. LG and MA designed and performed the clinical study. FR and KL planned the experiments and FR, KL and SB performed the experiments. FR, KL and ML planned the analysis. FR and KL

performed the analysis and prepared the figures. FR, KL and ML drafted the manuscript. All authors revised and approved the manuscript.

Competing interest

The authors declare no competing interest.

Supplementary Information

The supplementary information pdf-file contains:

Supplementary Figure S1. Serum levels of allergen-specific IgE and IgG4 are altered by AIT.

Supplementary Figure S2. Frequencies of basophils from allergic and non-allergic donors expressing various CLRs, TLRs and Fc receptors.

Supplementary Figure S3. Basophils express TLR2 and TLR4 on their surface.

Supplementary Figure S4. The frequencies of basophils expressing various CLRs and FcRs over time in the AIT study.

Supplementary Table S1. Number of donors included in the phenotypic analysis of basophils.

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Tables

Table 1. Donor characteristics and allergen mixtures used for AIT.

Donor	Sensitizations (Skin prick test)	Allergens for AIT	Gender and age
D1	BP, GP, CD, DD	3-tree, 5-grass, <i>Fel d</i>	M, 30
D2	BP, GP	3-tree, 5-grass	M, 30
D3	BP, GP, HDM, CD, DD	3-tree, 5-grass, <i>Der p</i>	F, 36
D4	HDM, CD, DD, MO	<i>Der p</i>	M, 23
D5	BP, MP, HDM, MO	3-tree, <i>Art v</i> , <i>Der p</i>	F, 41
D6	BP, GP, MP	3-tree, 5-grass, <i>Art v</i>	M, 27
D7	BP, GP, CD, DD, HD	3-tree, 5-grass, <i>Fel d</i>	F, 24
D8	BP, GP, MP, CD, DD	-	M, 25
D9	BP, GP, MP, DD	-	F, 30
D10	BP, GP	-	M, 23
D11	BP, HDM	-	F, 24
D12	BP, GP, HDM, CD, DD	-	F, 24
D13	BP, GP	-	F, 38
NA1	-	-	M, 30
NA2	-	-	F, 42
NA3	-	-	F, 41
NA4	-	-	M, 39
NA5	-	-	F, 51
NA6	-	-	M, 35

Abbreviations: F: female, M: male, NA: non-allergic.

Sensitizations: BP: birch pollen, CD: cat dander, DD: dog dander, GP: grass pollen, HD: horse dander, HDM: house dust mite, MO: mold, MP: mugwort pollen.

Immunotherapy: 3-tree: a mixture of tree pollen (alder, birch, hazel), 5-grass: a mixture of grass pollen (meadow foxtail, cocksfoot, meadow fescue, english ryegrass, timothy), *Art v*:

Mugwort (*Artemisia vulgaris*), *Der p*: house dust mite (*Dermatophagoides pteronyssinus*),

Fel d: cat dander (*Felis domesticus*).

Figure legends

Figure 1. Gating strategy. Viable cells were gated in FSC/SSC and basophils were further identified as lineage (CD3, CD14, CD19) and HLA-DR negative, and CD123 positive, cells (a). FMO controls were used to determine the net percent positive basophils expressing each PRR and FcR at the different time points. A representative example for Dectin-1 for t0 and t1y can be seen (b). The x-axis termed “Vacant” indicates that no antibodies were added for the FMO controls, in this case as a control for the Dectin-1-FITC staining.

Figure 2. Frequencies of basophils expressing various CLR (a), TLR (b) and Fc receptors (c). Flow cytometry was used to analyse the expression of PRRs and FcRs on basophils, and net percent positive cells were calculated based on the stained sample minus corresponding FMO control. n = 16-19 subjects per analysis (For detailed information see Supplementary table S1). Boxplots show the median (line), mean (+), 25-75 percentile (box) and minimum-maximum (whiskers).

Figure 3. AIT alters the CLR and FcR profile on human basophils. The frequencies of basophils expressing Dectin-1 (n = 4) (a), Dectin-2 (n = 5) (b) and FcγRII (n = 4) (c) vary with time upon AIT. Analysis was made with flow cytometry and net percent positive cells were calculated from the stained sample minus the FMO control sample. Statistical analysis was performed using Friedman test followed by Dunn’s multiple comparison test, * p < 0.05, ** p < 0.01, ns = not statistically significant. t0: before AIT, t8w: 8 weeks of AIT, t1y: one year of AIT.

Figure 4. The frequency of viable basophils in peripheral blood significantly increases after one year of AIT. Flow cytometric analysis was used to determine the frequency of basophils. Boxplots show the median (line), mean (+), 25-75 percentile (box) and minimum-maximum (whiskers), n = 7. Statistical analysis was performed using Friedman test followed

by Dunn's multiple comparison test, * $p < 0.05$, ns = not statistically significant. t0: before AIT, t8w: 8 weeks of AIT, t1y: one year of AIT.



